Essential Oil and Rumensin Affect Ruminal Fermentation in Continuous Culture

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Abstract

The combination of Rumensin® and essential oil could be beneficial for ruminal fermentation by suppressing protozoa and their associated methanogens, while maintaining normal rumen function. The objective of this study was to determine the effects of feeding Rumensin® and Cinnagar® (essential oil from cinnamon and garlic) in diets on ruminal fermentation characteristics. Four continuous culture fermenters were modified to retain protozoa (slower stirring and a special filter apparatus) and maintained at a liquid dilution rate of 7%/h and a solids dilution rate of 5%/h in 4 periods of 10 d each (7 d of adaptation, 3 d of sample collection) in a 4 X 4 Latin square design. Four dietary treatments (fed in 1 meal per day) were arranged in a 2 x 2 factorial: (1) control diet, 40 g of a 50:50 concentrate: forage (ground alfalfa hay) diet (40% neutral detergent fiber, 17% crude protein) containing no additive; (2) Rumensin® at 11g/ 909 kg of dry matter; (3) Cinnagar® at 0.0043% (DM basis); and (4) combination of Rumensin® and Cinnagar®. There were no effects of treatment on NDF or organic matter digestibility, concentrations of NH_3-N or total volatile fatty acid, or percentage of protozoal generic distribution. Rumensin® (main effect, no interaction) decreased ($P < 0.05$) molar percentages of acetate (62.6 vs 64.4) and valerate (1.78 vs 1.86); decreased acetate: propionate ratio (2.69 vs 3.04); and increased ($P < 0.05$) molar percentages of propionate (23.3 vs 21.3) and isovalerate (1.94 vs 1.67). Rumensin® increased ($P < 0.05$) the protozoa generation time (27.6 vs 21.6 h). Cinnagar® tended ($P = 0.11$) to increase molar percentage of isovalerate (1.77 vs 1.67) and decrease protozoa counts (14.9 vs. 18.5 x 10^3/ml). Rumensin® and Cinnagar® tended ($P = 0.06$) to interact for methane production (29.3, 22.4, 21.8, and 36.7 mmol/d, respectively). Under the conditions of our study, we did not detect an additive response for Rumensin® and Cinnagar® to either decrease protozoal counts or methane production.

Introduction

Essential oils (EO) are hydrophobic aromatic compounds that can be extracted from plants and have a high rate of antimicrobial activity against gram-positive and gram-negative bacteria, protozoa, and fungi (McIntosh *et al*., 2003). They are usually extracted by steam distillation or solvent extraction. Essential oils most likely have high antimicrobial activity due to their ability to disrupt microbial cell membranes which could affect any number of processes, such as electron transport, ion gradients, or translocation. Pure culture studies revealed that EO affected mostly ruminal hyperammonia - producing bacteria (*Peptostreptococcus anaerobius, Clostridium aminophilum,* and *Clostridium sticklandii*) and fungi, and as a result reduced the rate of deamination of amino acids (McIntosh *et al*., 2003). However, other *in vitro* studies using batch or continuous cultures have reported variable effects of EO on deamination of amino acids (Newbold *et al*., 2004; Busquet *et al*., 2006; Castillejos *et al*., 2006). When a combination of cinnamaldehyde (0.6 g/d) and eugenol (0.3 g/d) was fed *in vivo* (Cardozo *et al*., 2006), it decreased acetate and ammonia-N concentrations and increased propionate and led to accumulation of small peptides and amino acids in ruminal fluid. Although methane production was not measured in any of these studies, the change in the volatile fatty acid (VFA) profile indicates stoichiometrically that there may be decreased methane production, therefore insinuating that EO can inhibit methanogenesis. If methane production is impaired by EO, redox reactions and H_2 transfers could be inhibited as well. Redox reactions are needed in order for deamination to take place and monensin is often used for its effectiveness against H_2 - producing bacteria. From this information, it can be concluded that the combination of EO and monensin could inhibit deamination and consequently methanogenesis. There has been little research or attention placed on this particular subject, and as methane production becomes more of a growing issue, this research will become more important.

Another effect of EO that hasn't been extensively studied is the effect of EO on VFA metabolism (Calsamiglia *et al.*, 2007), despite evidence in some studies that there are no changes in fatty acid profiles in milk when diets are being supplemented with EO (Benchaar *et al*., 2006; Benchaar and Chouinard, 2009). Antibiotic ionophores and fish oil inhibit both methanogenesis and deamination; however, antibiotics are becoming increasingly unaccepted by the public in animal feeds and have even been outlawed in the European Union since 2006 (Calsamiglia *et al*., 2007). Therefore, essential oils are being considered as a potential candidate to control bacterial populations involved in ruminal biohydrogenation (Calsamiglia *et al*., 2007). Similarly, monensin has been shown to decrease the rate of ruminal biohydrogenation of unsaturated fatty acids *in vitro* (Fellner *et al*., 1997) and has increased the concentration of conjugated linoleic acid (CLA) in milk fat (AlZahal *et al*., 2008). In another study, ethanolic extracts of EO from 91 different Australian plants were examined. The results revealed that these EO inhibited the growth of *Clostridium proteoclasticum,* which resulted in high increases of CLA and vaccenic acid (VA) (Durmic *et al*., 2008). Cinnamaldehyde, an essential oil, has varying affects on

ruminal biohydrogenation and VFA metabolism. In one study where a continuous culture fermenter system was used, cinnamaldehyde was found to decrease biohydrogenation of C18: 2n-6 and C18: 3n-3 and to shift it from the *trans*-11 pathway to a secondary pathway, resulting in the accumulation of *trans*-10 C18:1 and trans-10, cis-12 CLA (Lourenço *et al*., 2008). This shift has been shown to cause a decrease in milk fat (Jenkins *et al*., 2008). Conversely, in another study (Benchaar and Chouinard, 2009), cinnamaldehyde had no effect on milk fat. This difference could be due to the absence of protozoa in the continuous culture. Protozoa do not contribute to biohydrogenation directly, but they could affect activity or populations of lipid-metabolizing bacteria by selective predation (Karnati *et al*., 2009b).

Because of increased public concern about global warming and the effect that methane gas can have on the environment, research is being directed towards decreasing methanogenesis, especially now that studies have shown that ruminants produce a lot of methane gas. Ruminal protozoa are also in close association with methanogens and therefore need to be suppressed (Williams and Coleman, 1992). To suppress protozoa by feeding fat, we must change the dietary conditions. Studies have shown that high grain diets versus high forage diets were more effective at suppressing the protozoa (Firkins, 1996). However, when Rumensin® is fed with higher grain diets, trans fatty acid production increases, which in turn causes the protozoal population to increase so it can reduce the formation of these fatty acids (Karnati *et al*., 2009a). An alternative, such as EO, can be used to keep this from occurring. There are variable effects on ruminal protozoa when EO is supplemented. When 2 g/d were given to dairy cows, there was no effect on the number of protozoa (Benchaar *et al*., 2006). When a mixture of cinnamaldehyde (180 mg/d) and eugenol (90 mg/d) were administered in diets of beef heifers, this increased the numbers of holotrichs and had no effect on entodiniomorphs. The variable results in these studies could be because of different types of EO and different EO concentrations or because of carryover effects on microbial populations in their Latin square designed project (28-d periods). The premise for this research is that protozoa adjust to Rumensin® by changing their membrane structure (Sylvester *et al*., 2009; Karnati *et al*., 2009a), but the additional challenge of EO along with Rumensin® might be needed to suppress protozoa long term while avoiding the need for large amounts of dietary unsaturated fatty acids that can cause a decrease in milk fat. Moreover, suppression of protozoa-associated methanogens may directly (with a specific inhibitor) increase the generation time of protozoa, which would make them less competitive and thus lower their biomass in the rumen. Therefore, combining Rumensin® plus EO could beneficially suppress protozoa and their associated methanogens.

Objectives

This study was designed to investigate the effects of an ionophore and an essential oil on rumen function, as well as their potential suppression of protozoa and the methane they produce. We hypothesized that combining Rumensin® plus Cinnagar® (an essential oil from garlic and cinnamon) could beneficially suppress protozoa and their associated methanogens without effecting rumen function.

Materials and Methods

Experimental Design

A modified dual flow continuous culture system designed to retain ruminal protozoa and simulate ruminal digestion and solid and liquid passage rates was used in these experiments (Karnati *et al*. 2009a). The fermenters were fed once daily a meal of 40 g of a 50:50 concentrate: forage diet (ground alfalfa hay, 38% NDF, 17% CP) containing either no additive, Cinnagar® provided by Provimi-North America (Lewisburg, Ohio) at 0.0043% DM basis, Rumensin® (Elanco Animal Health, Greenfield, Indiana) (11g/ 909 kg of DM), and Cinnagar® (0.0043% DM basis) + Rumensin® (11g/ 909 kg of DM) in a 4 X 4 Latin square design with 4 continuous culture fermenters over 4 periods of 10 d each (7 d of adaptation).

Continuous Culture Operation

The dual flow continuous culture system was based on the system described by Hoover *et al*. (1976) but with modified turnover rates compared with standard conditions (Noftsger *et al*., 2003) in order to increase the pool size of protozoa in the fermenters. The liquid dilution rate was maintained at 7%/h and the solid dilution rate at 5%/h by regulation of filtrate removal rates and buffer input. For each period, ruminal contents were taken from 2 separate cows maintained on a diet without Cinnagar® or Rumensin®, squeezed through 2 layers of cheesecloth, and inoculated into all 4 fermenters. As described in Karnati *et al*. (2009a), a multi-stage filter system was used on the filter pumps to retain protozoa so that they would pass mostly with the overflow (4%/h). The buffer added to the fermenters maintained the pH between 6.2 and 6.7 over the feeding cycle. Agitation was set at 50 rpm, and temperature was maintained at 39°C. Flow rates were determined by weighing the solid and liquid effluents once a day during the adaptation period and adjusted as required.

Sample Collection and Analyses

On d 5, 10 % enriched $(^{15}NH_4)_2SO_4$ was added to the fermenters for use as a microbial marker. A sample of effluent was taken prior to the primed, continuous infusion for background ^{15}N. A sample of the daily effluent (20%) was taken on d 8, 9, and 10 in each period and composited by fermenter for analysis. Freeze-dried effluent samples were analyzed for N, NDF, organic matter (OM), and ammonia using standard methods (Noftsger *et al*., 2003). Digestibilities of OM and NDF were calculated from the analyzed concentrations of these components in the diet and in the effluent and the amount of effluent per day. The amount in the effluent per day was subtracted from the intake and divided by the intake to calculate digestibility. Ammonia nitrogen was determined according to the procedures described in Karnati et al. (2009a), and peptide-N was calculated by subtracting ammonia-N from total N. Bacterial and effluent samples and ammonia samples were analyzed for ^{15}N by the Stable Isotope Laboratory at Pennsylvania State University. An aliquot of the effluent sample was strained through 4-layers of cheesecloth. The filtrate was acidified using 3 ml of 6 *N* HCl per 50 ml of filtrate to stop fermentation prior to analysis for VFA (Firkins *et al*., 1996), samples were centrifuged, and the supernatant was injected into a gas chromatograph. An aliquot of the effluent sample and the fermenter contents were fixed in formalin, and protozoal counts were determined. During the collection period, gas-impermeable Mylar bags were attached to a steel tube attached to the head plate for collection of total fermentation gases. A sample of gas was analyzed for methane using a gas chromatograph (Karnati *et al*., 2009a) and multiplied by total daily gas production to determine production rates of methane.

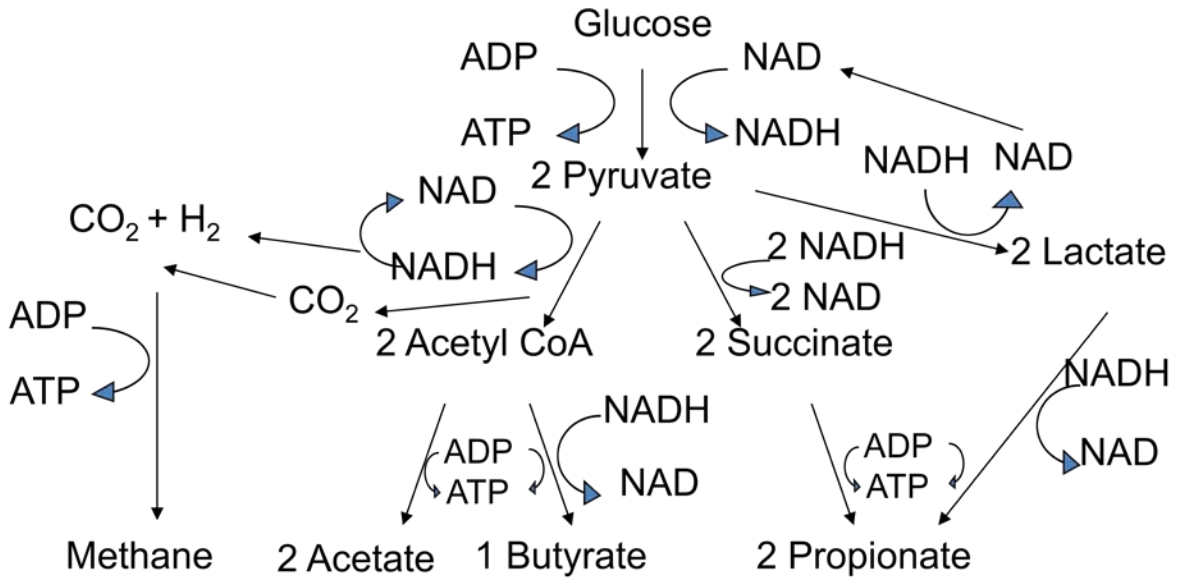
Results and Discussion

The VFA profile for Rumensin® changed just as it was expected based on previous studies (Table 1). Rumensin® (main effect, no interaction) decreased ($P < 0.05$) molar percentages of acetate and valerate from 64.4 to 62.6 and 1.86 to 1.78, respectively. Rumensin® also increased ($P < 0.05$) molar percentages of propionate and isovalerate from 21.3% to 23.3% and 1.67% to 1.94%, respectively. Consequentially, the decrease in acetate and the increase in propionate caused a decrease in the acetate: propionate ratio from 3.04 to 2.69.

The VFA profile for Cinnagar® was not consistent with the previous study conducted by Cardozo *et al*. (2006) where acetate was decreased. In our study, Cinnagar® tended ($P = 0.11$) to increase isovalerate from 1.67 to 1.77, but had no other significant effects on the VFA profile. Although the study conducted by Cardozo *et al*. (2006) did not measure methane production, the change in VFA profile was stoichiometrically consistent with a decrease in methane production. Therefore, because Cinnagar® failed to change the VFA profile in our study, there was no significant decrease in methane production when Cinnagar® was added to the diets.

Even though there was no statistical change in methane production when Rumensin® and Cinnagar® were added alone, there tended (P = 0.06) to be a decrease in methane production for both diets which is stoichiometrically consistent with decreased acetate and increased propoionate and peptide N, indicating a decrease in deamination with accumulation of small peptides and amino acids. When methane production is decreased or stopped, production of propionate is increased and acetate and butyrate decrease because of a build-up of NADH. This build-up slows down the oxidation of pyruvate to acetyl CoA, thus decreasing methane production as shown in Figure 1.

Figure 1. Glycolysis pathway.



Yet, in the Rumensin® and Cinnagar® combination diet, there tended (P = 0.06) to be an interaction for methane production, increasing from 29.3 mmol/d to 36.7 mmol/d, which is the opposite result of what the pathway would suggest. There were no apparent problems in the methane data or with the operation of gas analysis, so the cause of this interaction is assumed to not be from human or experimental error.

One hypothesis for this result is that there was a shift in the type of bacteria present in the ruminal content when the combination diet was fed, which in turn caused more hydrogen to be produced. The increase in hydrogen would increase the methane production. For example, the major ruminal bacteria, *ruminococcus albus*, produce acetate, H_2, and succinate, while *fibrobacter succinogens* produce other products rather than H_2. Therefore, the shift among different ruminal bacteria could possibly increase the methane production. Additionally, the meta analysis by Patra (2010) showed that concentrations of total VFA and propionate changed

linearly and positively with changes in methane production by EO. However, acetate production and the acetate:propionate ratio increased linearly with increasing inhibition of methane by EO in *in vitro studies*.

There were no effects of treatment on NDF or organic matter (OM) digestibilities and concentrations of NH_3-N (Table 2). However, the difference between NDF and OM digestibilities were rather large. Typically *in vivo*, OM digestibility is larger than for NDF. *In vitro* NDF digestibility can be a larger percentage than OM digestibility but typically not as large as what was found in this study (73.6 vs. 46.3 in the control diet). There are a few possible causes for this finding. One possibility is that the OM digestibility was underestimated due to the buffer solution that was added to the fermenters. The buffer has minerals that when added to the samples become an additional source of organic matter. Another possibility is that when freeze-dried samples were washed after they were collected, the fine powder that is associated with NDF was also washed away. This would result in an overestimation of NDF digestibility. Finally, accumulation of feed at the bottom of the stirring jar could have affected the percentage of NDF. This accumulation would result in an overestimation of NDF. Rumensin also caused an increase ($P < 0.05$) in peptide-N, indicating less deamination of amino acids.

Both Rumensin® and Cinnagar® were expected to increase generation time and decrease protozoal counts. Rumensin® and Cinnagar® disrupt the pellicle or outside layer of the protozoa, which causes increases in generation time and decreases in protozoa count to occur. Rumensin® increased ($P < 0.01$) the protozoa generation time from 20.8 to 27.0 h, and Cinnagar® tended to decrease ($P = 0.11$) the protozoa counts from 19.2 x 10^3/ml to 13.3 x 10^3/ml. Cinnagar® tended ($P = 0.12$) to increase *Entodinium*, but there were no other effects on generic distribution of protozoa (Table 4).

Although there were defects in the fermenter trials, our goal was to look at the relative effects of these treatments versus the absolute effects which are more conducive in an *in vitro* study. Any errors become negligible because we were looking at the comparison between values rather than the absolute values. An *in vivo* study might produce different results and is harder to control, especially when factors that were not predicted arise. Rumensin® has been shown to cause a decrease in milk fat, as well as prevent possible metabolic diseases like ketosis (Duffield *et al*., 1998), but a combination treatment between monensin and an essential oil could produce detrimental effects to milk production and composition. Therefore, additional research is warranted.

Conclusions

Under the conditions of our study, we did not detect an additive response for Rumensin® and Cinnagar® to either decrease protozoal counts or methane production. Additionally, the generation time was expected to increase with the Rumensin® and Cinnagar® diet, but this did not occur in our study. The fermenters may have had an effect on these results. Further explanation for these results could come from the nitrogen flow data which has not been analyzed completely at this time.

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Table 1. Fermentation characteristics in continuous cultures fed diets without or with Rumensin® and Cinnagar®.

	Rumensin®[1]							
	-	-	+	+		Contrasts[2]		
	Cinnagar®[1]							
	-	+	-	+	SEM	Rum	Cin	Rum*Cin
VFA, mmol/L	53.3	51.4	49.1	53.2	3.12	0.67	0.68	0.28
Individual VFA (mol/100 mol)								
Acetate	64.4	64.8	62.6	62.8	0.39	<0.01	0.42	0.86
Propionate	21.3	20.9	23.3	23.3	0.37	<0.01	0.59	0.66
Isobutyrate	0.71	0.68	0.58	0.72	0.10	0.67	0.64	0.42
Butyrate	10.1	10.0	9.84	9.45	0.25	0.14	0.36	0.54
Isovalerate	1.67	1.77	1.94	2.07	0.09	<0.01	0.11	0.81
Valerate	1.86	1.85	1.78	1.67	0.04	0.02	0.17	0.24
Acetate: Propionate	3.04	3.12	2.69	2.71	0.06	<0.01	0.46	0.64
Methane (mmol/d)	29.3	21.8	22.4	36.7	6.02	0.42	0.50	0.06

[1]Diets consisted of 40 g/d of a 50:50 concentrate: forage containing Rumensin® at 11g/ 909 kg or Cinnagar® at 0.0043% on a dry matter basis.
[2]Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin).

Table 2. Digestibilities of NDF and OM and concentrations of ammonia nitrogen and peptide nitrogen in continuous cultures diets without or with Rumensin® and Cinnagar®

	Rumensin®[1]							
	-	-	+	+		Contrasts[2]		
	Cinnagar®[1]							
	-	+	-	+	SEM	Rum	Cin	Rum*Cin
NDF digestibility (%)	73.6	74.5	73.8	73.3	1.61	0.76	0.89	0.66
Apparent OM digestibility (%)	46.3	46.0	46.8	44.7	1.62	0.83	0.50	0.59
NH_3-N (mg/dl)	15.0	14.9	15.0	15.6	0.36	0.40	0.51	0.36
Peptide N (mg/dl)	7.47	7.85	8.41	8.44	0.41	0.05	0.52	0.58

[1]Diets consisted of 40 g/d of a 50:50 concentrate: forage containing Rumensin® at 11g/ 909 kg or Cinnagar® at 0.0043% on a dry matter basis.
[2]Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin).

Table 3. Protozoal counts and generation (Gen) time in continuous cultures diets without or with Rumensin® and Cinnagar®.

	Rumensin®[1]				Contrasts[2]			
	-	-	+	+				
	Cinnagar®[1]							
	-	+	-	+	SEM	Rum	Cin	Rum*Cin
Total, 10^3 x ml^{-1}	19.2	13.3	17.7	16.5	1.91	0.68	0.11	0.27
Gen time, h[3]	20.8	22.3	27.0	28.2	1.21	<0.01	0.29	0.88

[1]Diets consisted of 40 g/d of a 50:50 concentrate: forage containing Rumensin® at 11g/ 909 kg or Cinnagar® at 0.0043% on a dry matter basis.

[2]Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin).

[3]Total pool size of cells in fermenter / flow of cells in effluent.

Table 4. Protozoal generic distribution in continuous cultures diets without or with Rumensin® and Cinnagar®

	Rumensin®[1]				Contrasts[2]			
	-	-	+	+				
	Cinnagar®[1]							
Generic distribution, %[3]	-	+	-	+	SEM	Rum	Cin	Rum*Cin
Entodinium	90.8	93.7	90.2	92.1	4.02	0.42	0.12	0.53
Isotrichidae[4]	5.93	5.02	6.65	5.15	1.40	0.73	0.34	0.80
Diplodiniinae[5]	1.93	0.83	1.90	1.83	0.47	0.35	0.26	0.32

[1]Diets consisted of 40 g/d of a 50:50 concentrate: forage containing Rumensin® at 11g/ 909 kg or Cinnagar® at 0.0043% on a dry matter basis.

[2]Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin).

[3]% of total counts. *Ophryoscolex*, *Epidinium*, and other genera were periodically detected but not listed.

[4]Family, including the genera *Dasytricha* and *Isotricha.*

[5]Subfamily, including the genera *Diplodinium, Eudiplodinium, Enoploplastron, Metadinium, Ostracodinium* and *Polyplastron.*